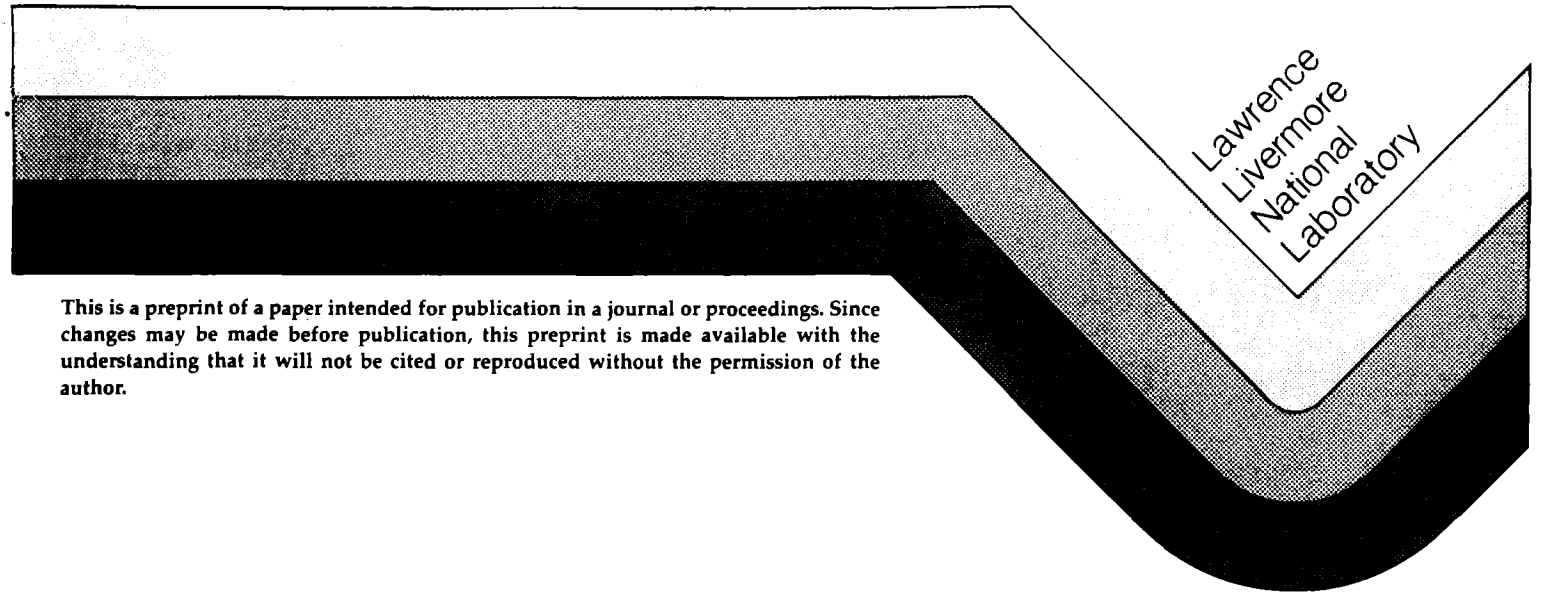
UCRL- JC-109907
PREPRINT

Bioremediation of Trichloroethylene Under Simulated In Situ Conditions: A Resting Cell Methanotrophic Microbial Filter Approach

R.T. Taylor, W.B. Durham, A.G. Duba, M.L. Hanna, K.J. Jackson, M.C. Jovanovich, R.B. Knapp, J.P. Knezovich, N.N. Shah, D.R. Shonnard, and A.M. Wijesinghe

This paper was prepared for submittal to Science Magazine

February 26, 1992



Bioremediation of Trichloroethylene Under Simulated *In Situ* Conditions: A Resting Cell Methanotrophic Microbial Filter Approach

Robert T. Taylor, W. B. Durham, A. G. Duba, M. L. Hanna, K. J. Jackson, M. C. Jovanovich, R. B. Knapp, J. P. Knezovich, N. N. Shah, D. R. Shonnard, and A. M. Wijesinghe

[submitted to *Science*, February, 1992]

R. T. Taylor, M. L. Hanna, N. N. Shah, D. R. Shonnard, Lawrence Livermore National Laboratory, Biomedical Sciences Division, Livermore, CA 94550.

A. G. Duba, W. B. Durham, K. J. Jackson, R. B. Knapp, A. M. Wijesinghe, Lawrence Livermore National Laboratory, Earth Sciences Department, Livermore, CA 94550.

J. P. Knezovich, M. C. Jovanovich, Lawrence Livermore National Laboratory, Environmental Sciences Division, Livermore, CA 94550.

ABSTRACT

We are testing and developing an *in situ* microbial filter technology for remediating migrating subsurface plumes contaminated with low concentrations of trichloroethylene (TCE). Our current focus is the establishment of a replenishable bioactive zone (catalytic filter) along expanding plume boundaries by the injection of a representative methanotrophic bacterium, *Methylosinus trichosporium* OB3b. We have successfully demonstrated this microbial filter strategy using emplaced, attached resting cells (no methane additions) in a a 1.1-meter flow-through test bed loaded with water-saturated sand. Two separate 24-hour pulses of TCE (109 ppb and 85 ppb), one week apart, were pumped through the system at a flow velocity of 1.5 cm/hr; no TCE (<0.5 ppb) was detected on the downstream side of the microbial filter. Subsequent excavation of the wet sand confirmed the existence of a TCE-bioactive zone 17 days after it had been created. An enhanced longevity of the cellular, soluble-form methane monooxygenase produced by this methanotroph is a result of our laboratory bioreactor culturing conditions.

Groundwater aquifers at many industrial and defense-related sites are contaminated with one- and two-carbon, volatile, chlorinated aliphatic hydrocarbons (*e.g.*, TCE), many of which are suspected carcinogens (1-4). Due to the natural flow of groundwater and dispersive transport processes, aquifer contamination at such sites has usually resulted in large, dilute, migrating plumes that can extend tens of meters in depth (5). The contaminants are present in the aqueous phase and they are sorbed on the mineral phases that constitute a heterogeneous soil or rock. These physical characteristics of aquifer contamination make groundwater remediation to the concentrations mandated by the EPA (≤5 ppb for TCE) a challenging problem.

Pump-and-treat is currently the standard method for remediating volatile organic compounds. In this process, the plume is penetrated by a number of wells, contaminated groundwater is extracted from the wells, and the water is treated and disposed of at the surface (6). Pump-and-treat has great uncertainty in the ultimate level of decontamination, the time required to achieve this level, and the permanence of the decontamination (7,8). The main cause of these uncertainties is the highly heterogeneous nature of the subsurface medium, which creates preferential flow paths for the extracted fluids. Less permeable subsurface regions receive less remediation and remain as sources of residual contamination to recontaminate the cleaned-up regions.

In situ microbial bioremediation of aquifers contaminated with volatile organic compounds has received increasing attention as an alternative to pump-and-treat (8-10). It has the potential advantages that many common organic contaminants can be biodegraded to innocuous compounds by naturally occurring microorganisms, that it is a contaminant-destructive as opposed to contaminant-relocative process, and that it is carried out *in situ*, obviating the need for disposal of the treated groundwater. Unfortunately, clear-cut proof that biodegradation of the undesired organic contaminant has taken place in an aquifer has been difficult to substantiate (11), even for TCE added in known amounts to a very small, shallow aquifer (12).

The usual approach to *in situ* aquifer bioremediation is to pump a suite of nutrients into the subsurface to stimulate the growth of indigenous bacterial populations (8-10,12,13). Ideally, the resulting increase in biomass causes the desired biodegradation at an acceptable rate. However, three complications arise: (1) heterogeneous permeability of the subsurface environment makes it difficult to deliver nutrients throughout the contaminated plume, (2) nutrient pumping often causes preferential growth near the injection wells and can lead to biofouling, and (3) the biotransformation of chlorinated hydrocarbons is generally a cometabolic phenomenon.

Cometabolism, the fortuitous microbial transformation of compounds such as chlorinated aliphatic hydrocarbons, cannot provide the energy or carbon needed for cell division. Growth stimulation, therefore, depends on another carbon source that may competitively inhibit the contaminant biodegradation. In addition, the stimulation of aerobic microbial growth utilizes

dissolved oxygen that is needed for contaminant cometabolism. Methanotrophs, which are ubiquitous aerobic bacteria that can be cultured on methane as a sole carbon source, typify this situation. TCE cometabolism has been reported for methanotrophic mixed cultures (14-25) and pure cultures (23,26-31) under an assortment of laboratory test conditions. With one exception (21), the growth substrate (methane) was found to be a competitive inhibitor of methanotrophic TCE degradation in laboratory studies (20,28,31) and an aquifer field study (12). This is not surprising, because it has been known for some time that the methane monooxygenases (MMOs) produced by methanotrophs can exhibit oxidative activities with a broad range of organic substrates, particularly alkene epoxidations (32,33). By analogy to the MMO-catalyzed conversion of propene to propene oxide, it is generally assumed (15,25,26) that methanotrophs initially biotransform TCE to its corresponding epoxide, which is highly reactive and rapidly hydrolyzed (half-life ~ 12 sec) in aqueous systems (34,35).

Microbial Filter Strategy

As an alternative to the approach based on nutrient stimulation, we are investigating an *in situ* microbial filter strategy using resting (*i.e.*, non-dividing) cells. In this strategy, microorganisms are injected into the subsurface ahead of migrating contaminant plumes. The inocula, obtained from surface bioreactors, could be either a pure strain or a mixture of bacteria that collectively possess the required metabolic properties. We anticipate that a portion of the injected microorganisms attach to the soil or rock and form a zone of enhanced biodegradative activity. Ideally, contaminated groundwater flows into this zone, the attached microbial population biodegrades the contaminant(s) at a rate that keeps pace with the rate of transport, and groundwater then exits clean. Such a strategy also allows for concurrent treatment of the contaminant source by microbial inoculation and for the emplacement of multiple biofilters across the expanding dimensions of the plume to accelerate the remediation process. This combined *in situ* microbial filter plus source treatment approach is based, in part, on techniques that have been employed for microbial enhanced oil recovery (36).

To test this biofilter strategy in the laboratory, we developed a meter-scale test bed (Fig. 1a). The internal dimensions of the frame containing the test bed were 1.1 m in length, 0.4 m in height, and 0.1 m in width. Flow of water in the test bed was horizontal, along the long dimension, and was strictly one-dimensional. Except for the three glass inspection windows shown in Fig. 1b, the inside surfaces of the frame were nickel metal, mostly pure nickel foil covering nickel-plated stainless steel. The frame was filled with water and then with a commercially available sand. It was a high-purity quartz sand with trace amounts of hematite, illite, and calcite. The grain size distribution was narrow, with 84% of the grains having a diameter between 0.106 and 0.25 mm. The sand pack was completely saturated (no visible gas bubbles), and had a bulk permeability of approximately 8.5 Darcys and a porosity of 0.32 ± 0.02. Once the test bed was sealed, a horizontal flow of 10 mM Higgin's phosphate buffer (37) was initiated and maintained at a constant rate of 200 mL/h (flow velocity $\approx$ 1.5 cm/h, a typical value for the flow of natural groundwater). All experiments were conducted at 21-22°C in a temperature-controlled room. A colored tracer (phenol red) and time-lapse photography through the glass windows revealed a 25% increase in permeability from the top to the bottom of the test bed, but the increase was roughly constant, without any undesirable "fingering" of flow, for example. Tracer tests also showed convincingly that there were no permeability gradients in the horizontal plane and that there were no short circuits for flow through the bed.

Fluids within the wet sand pack were sampled through side-wall ports (Fig. 1a) attached to the ends of porous, 6-mm outer diameter, thick-walled fritted nickel tubes that extended across the full 0.1-m width of the sand pack. The nickel tubes had roughly the same porosity and permeability as the sand pack and a pore size of approximately 20 μm. Tracer tests demonstrated that fluids taken from the ports were sampled uniformly across the sand pack.

Methylosinus trichosporium OB3b was selected as the prototype methanotroph for these tests for several reasons. First, it is one of the two best-characterized methane-oxidizing bacterial strains currently available. Second, it can be easily grown in bioreactors at 30°C (doubling-time ~7 h) to yield high densities of bacteria that contain only the soluble form (sMMO), as opposed to the

membranous particulate form (pMMO) of intracellular methane monooxygenase (37). Third, *M. trichosporium* OB3b cells producing sMMO are among the most active methanotrophs with respect to TCE biodegradation catalysis (27,28,30,31). In related experiments, fresh cells containing sMMO convert propene to propene oxide at rates of 100-150 nmol/min/mg dry cell wt at 30°C in the presence of 20 mM formate as an electron donor (37). With 0.5 μmol of unlabeled or [1,2-^{14}C]TCE (2,000 cpm/nmol) substituted for propene in the same standard assay mixture (37), the initial steady-state rate of biotransformation (measured either by TCE disappearance or the appearance of non-volatile water-soluble radioactive products) is routinely 40-60 nmol/min/mg dry cell wt (39). We agree with other investigators (27,28,30,31) that for *M. trichosporium* OB3b, whole-cell biodegradation of TCE is decidedly a catalytic property of the sMMO as opposed to the pMMO. The TCE biotransformation rate for cells containing sMMO is 75-100 times faster than for cells containing pMMO, under both saturating and limiting concentrations of the TCE substrate (39).

Biodegradation Experiments

At the start of the experiments *M. trichosporium* OB3b bacteria, which had been batch-cultured in a 5-L bioreactor to produce sMMO (37), were harvested by centrifugation, washed, and resuspended in Higgin's phosphate buffer (pH 7.0) (37). As detailed in the legend to Fig. 1a, they were then pumped into the test bed to create an attached biofilter that was about 0.1 m thick in the flow direction. Cell-count-balance data revealed that 9% of the injected bacteria attached to the sand. After this inoculation, a 24-hour pulse of TCE (528 μg) at a concentration of 109 ppb in Higgin's phosphate was pumped into the test bed at the inlet port. Analyses by gas chromatography (40) of 1-mL aliquots withdrawn from ports immediately ahead of the emplaced microbial filter showed the arrival of the TCE pulse (Fig. 2), whereas no TCE was detected immediately downstream of the filter (Fig. 3). The expected TCE arrival times for no TCE biodegradation were simulated by using a one-dimensional mathematical model for advective-dispersive transport in a porous medium with linear equilibrium sorption (41). The data in Figs. 2 and 3 indicate complete biodegradation of the TCE pulse (>99%). The experiment was repeated 7 days later with a second

24-hour TCE pulse (316 μg) at a concentration of 85 ppb, pumped through the originally emplaced microbial filter. The result was comparable to that of the first experiment in that no TCE could be detected downstream of the biofilter (Figs. 4 and 5).

These two biofilter experiments were preceded by two mass-balance control experiments in which 24-hour, ~100-ppb pulses of TCE were passed through the test bed before the *M. trichosporium* OB3b cells were introduced. The sequential appearance of the TCE pulse at each port of the middle row (row "C" in Fig. 1a) along the entire 1.1-m length of the test bed is in vivid contrast to the quantitative removal of the TCE pulse by the attached microbial filter (Fig. 6). Total recoveries of TCE at the outlet for the two mass-balance control experiments were 76 and 84%, respectively.

Permeability measurements of the sand pack were made before the TCE mass-balance control experiments and again after the experiments with the emplaced biofilter. During this interval of approximately 6 weeks, fluid had been flowing continuously through the test bed, except during inoculation. No measurable change in permeability was observed, confirming the absence of any biofouling. Likewise, none was predicted, based on our calculations that in the microbial filter zone only 3% of the surface area of the sand grains were covered by the attached cells.

After the post-experimental permeability measurements, 17 days after the bacteria were emplaced, the test bed was disassembled and the sand pack was systematically sampled in the Plate I area (Fig. 1a), which included the injected biofilter zone. Measurements of the attached cell population and the remaining levels of whole-cell sMMO catalytic activity (for labeled TCE) were performed with these samples (Fig. 7). The maximum attached cell population density was on the order of 10^8 cells/g of dry sand and the maxima were approximately centered around the column of ports at which they were injected; a small amount of downstream spreading can be inferred. The residual whole-cell sMMO activity averaged 0.2 nmol of TCE oxidized/min/g of dry sand: it was skewed somewhat downstream from the remaining attached bacterial population. This suggests some preferential loss of catalytic activity in the upstream portion of the microbial filter, where most of the TCE biodegradation likely occurred. No TCE or other volatile halogenated

compounds were detected when representative samples of the sand pack from throughout the excavated test bed were heated to 60°C and analyzed by gas chromatography.

The results presented in Figs. 2-7 demonstrate that a microbial filter was established by injecting resting cells and that this filter removed ~100-ppb levels of TCE to below a detectable limit of 0.5 ppb. Yet TCE removal alone does not constitute proof that this contaminant disappearance is primarily due to *M. trichosporium* OB3b biodegradation. To address this point, an aliquot of the cells utilized for injection was set aside and later challenged with a low level of [1,2-^{14}C]TCE. The resting-stage age of these cells was identical to that of the bacteria that earlier were attached to the sand pack and subsequently excavated and detached (Fig. 7). In Fig. 8, the concentration of [1,2-^{14}C]TCE (131 ppb) was chosen to be similar to the previous test bed pulses of unlabeled TCE. Moreover, the total incubation period (Fig. 8) was selected to span the time required for a TCE wave to traverse Plate I of the test bed (Figs. 2, 4, and 6). It can be seen (Fig. 8) that 80-85% of the [1,2-^{14}C]TCE was biotransformed to water-soluble products. About 10% of the radiolabeled TCE that was added to the control incubation vials could not be recovered from the organic phase (42); this may account for the apparently incomplete cellular conversion. At 17 and 35 hours, all of the water-soluble ^{14}C-product material was non-volatile (stable to freeze drying). A preliminary analysis of this mixture showed that it consisted of [^{14}C]bicarbonate, [^{14}C]formate, and several other components (39). Also, a small amount of the added ^{14}C (5-8%) was firmly associated with the cell pellet. Similar findings have been reported by others, when [1,2-^{14}C]TCE was incubated with either a mixed (15) or pure (26,31) methanotrophic culture. Collectively the data in Fig. 8, in combination with the results in Figs. 2-7, strongly support the view that virtually complete biodegradation of the TCE pulse in the test bed was effected by the microbial filter.

Scale-Up to Field Site

It is interesting to note that the 1.1-m length of our flow-through test bed system (Fig. 1) does not differ markedly from the distances over which the partial biodegradation of several chlorinated ethenes was achieved in a recent field study (12). At a carefully designed and instrumented field

site located in Mountain View, CA, McCarty and his colleagues evaluated *in-situ* methanotrophic bioremediation in a shallow, semiconfined aquifer that was about 1.5 m thick and consisted of fine- to coarse-grained sands and gravel. Dissolved methane and oxygen were injected into the aquifer in alternating pulses to stimulate the growth of indigenous methanotrophs. Monitoring wells were located at 1, 2.2, and 3.8 m and an extraction well was situated 6 m downstream from the injection well. After several weeks of gas injection, biostimulation of the aquifer was achieved, but the methane-oxidizing bacteria grew preferentially within 2 m of the injection well (12a). When TCE (97 ppb and later 51 ppb) was then injected, along with more methane and oxygen, the maximum biodegradation attained within 2 m of the downstream travel was only 20-30% (12). Although our objective with a resting cell microbial filter is to eliminate growth-substrate competition with contaminant biodegradation, as well as growth-dependent oxygen exhaustion, the periodic pumping of a limited supply of methane plus air could be one method to replenish a spent biofilter. The above field study suggests that it promotes localized methanotrophic bacterial growth. The more obvious replenishment method would be simply to inject additional bioreactor grown-cells.

In contemplating a scale-up of our resting cell methanotrophic filter to a field site near the Lawrence Livermore National Laboratory, a bioactive zone 50 m across, 5 m high, and 1 m thick is estimated to be adequate to capture a migrating contaminant plume. The aquifer water has a pH of about 7.5, an electrical conductivity and ionic strength about the same as 10 mM Higgin's phosphate buffer, and it contains TCE (~100 ppb) as the predominant contaminant. In planning such a scale-up, three fundamental considerations are crucial with respect to the bacteria: the efficiency of bacterial attachment in the porous subsurface media, the longevity or stability of the whole-cell sMMO catalytic activity, and the absolute quantity of TCE that a given number or mass of cells can biotransform. Since the attachment efficiency will determine the amount of biomass that must be grown to create the filter, work is in progress to define some of the key variables that influence this process for the mineralogy characteristic of this subsurface aquifer. Much progress, however, has already been made recently toward maximizing the stability of *M. trichosporium*

OB3b sMMO in resting cells. The bioreactor culture medium and cell harvest time have a profound influence on the subsequent sMMO longevity. If the cells are grown on standard Higgin's medium (37a) and harvested during the logarithmic phase, the whole-cell sMMO half-life is only ~3 days; if they are collected in the slow growth phase (37a), it is increased to ~6 days. But if they are cultured under modified conditions and harvested as described (37) for the test bed experiments, the sMMO half life, using [1,2-^{14}C]TCE as the substrate, is greatly extended to ~35 days (Fig. 9). Figure 9 depicts the resting cell storage data for two separate batch cultivations. Similar results have been obtained when the cells were cultured in the same manner and then stored in small tubes of buffer-saturated quartz sand. Although the cause of this markedly enhanced stability is currently unknown, the consistent biphasic shape of the data (Fig. 9) suggests that the whole-cell sMMO is fully retained or spared, until some other intracellular component, perhaps NADH-regenerating or energy-yielding, is consumed.

Several laboratories have reported that non-dividing resting cell suspensions of both mixed and pure methanotrophic cultures are irreversibly damaged during the catalysis of TCE degradation (23-25,31 and references therein). Cellular TCE biotransformation is accompanied by a reduced cell viability and a lowered subsequent rate of methane or TCE oxidation. These effects are thought to be imparted by the hypothetical immediate product, TCE-epoxide, which can readily form covalent adducts with intracellular macromolecules. By repeatedly exposing a mixed methanotrophic cell suspension (resting state) at 21°C to 15-ppm TCE and measuring the disappearance of TCE after each addition until the MMO activity ceased, Alvarez-Cohen and McCarty obtained a finite TCE transformation capacity of 0.036 mg/mg of dry cell wt (24). From repeated exposures of *M. trichosporium* OB3b at 21°C (no added formate) to 13.1 ppm of [1,2-^{14}C]TCE (2,000 cpm/nmol), with centrifugal reisolation of the cells for each successive addition, a finite transformation capacity of 0.24 mg/mg of dry cell weight (±0.03 s.d.) was found for three separate experiments (39). Either wide differences exist among the capacities of methanotrophs for TCE transformation or our apparently much greater capacity merely reflects that only ~20% of their mixed culture cell mass (24) had functional MMO activity with TCE.

For the application of a resting methanotrophic filter to the LLNL field site mentioned above, the use of a pure culture with much greater MMO longevity and a much higher TCE transformation capacity may offer a significant advantage over the use of a mixed culture. At an estimated flow of ~0.38 x 10^6 L/day through the filter, the TCE load would be ~38 g/day. Using the bulk density of 1.69 x 10^3 kg/m^3 for the sand in the biofilter area and a *M. trichosporium* OB3b attached cell density of 1 x 10^8/g of sand, the filter biomass requirement would correspond to 25 kg. Based on a TCE capacity of 0.24 mg/mg of cell wt, this filter could in principle biodegrade the TCE flowing through the filter region for 158 days. Given these parameters, the lifetime of such a microbial filter for this particular field site would most likely be dictated by the longevity of the intracellular sMMO, the duration of microbial attachment, and additional factors. For plumes with greater TCE loading rates, however, the finite transformation capacity of the freshly cultured bacteria is a factor of comparable and perhaps dominant importance when compared to enzyme longevity and attachment duration. Recent results with a mixed methanotrophic culture (24) and our own preliminary findings with *M. trichosporium* OB3b (39) indicate that the transformation capacity can be increased by at least 2-fold with the addition of an electron donor to facilitate more intracellular NADH regeneration. In summary, we envisage a resting-cell microbial filter strategy as having the most promise for aquifers with relatively rapidly moving dilute plumes of contaminants such as the chlorinated ethenes and chloroform, where the operational advantage over conventional methods is greatest.

REFERENCES AND NOTES

1. U.S. EPA, *The Occurrence of Volatile Synthetic Organic Chemicals in Drinking Water*, Science and Technology Branch, Criteria and Standards Division, Office of Drinking Water, Dec. (1981).
2. J. J. Westrick, J. W. Mello, and R. F. Thomas, *J. Am. Water Work Assoc.* 5, 52 (1984).
3. P. F. Imfante and T. A. Tsongas, *Environ. Sci. Res.* 25, 301 (1982).
4. U.S. EPA, *National Revised Primary Drinking Water Regulations: Volatile Synthetic Organic Chemicals*, Fed. Reg. 49, 24329 (1984).
5. D. W. Layton, Ed., *Baseline Public Health Assessment for CERCLA Investigations at the LLNL-Livermore Site, Technical Report No. UCAR-10279*, Lawrence Livermore National Laboratory, Livermore, CA, June (1989).
6. H. H. Russell, J. E. Matthews, and G. W. Sewell, *Remediation* 1, 167 (1990).
7. C. C. Travis and C. B. Doty, *Environ. Sci. Technol.* 24, 1464 (1990).
8. K. H. Baker and D. S. Herson, *Geomicrobiol.* 8, 133 (1991).
9. J. T. Wilson, L. F. Leach, M. J. Hensen, and J. N. Jones, *Ground Water Monit. Rev.* Fall, p. 56 (1986).
10. M. D. Lee, J. M. Thomas, R. C. Borden, P. B. Bedient, C. H. Ward, and J. T. Wilson, CRC *Crit. Rev. Environ. Control* 18, 29 (1988).
11. E. L. Madsen, J. L. Sinclair, and W. C. Ghiorse, *Science* 252, 830 (1991).
12. (a) P. V. Roberts, G. D. Hopkins, D. M. Mackay, and L. Semprini, *Ground Water* 28, 591 (1990); (b) L. Semprini, P. V. Roberts, G. D. Hopkins, and P. L. McCarty, *Ground Water* 28, 715 (1990); (c) L. Semprini, G. D. Hopkins, P. V. Roberts, D. Grbic-Galic, and P. L. McCarty, *Ground Water* 29, 239 (1991); (d) L. Semprini and P. L. McCarty, *Ground Water* 29, 365 (1991).
13. P. E. Flathman, D. E. Jerger, and L. S. Bottanley, *Ground Water Monit. Rev.* 9, 105 (1989).
14. J. T. Wilson and B. H. Wilson, *Appl. Environ. Microbiol.* 49, 242 (1985).

15. M. M. Fogel, A. R. Taddeo, and S. Fogel, *Appli. Environ. Microbiol.* 51, 720 (1986).

16. D. B. Janssen, G. Grobben, R. Hoekstra, R. Oldenhuis, and B. Witholt, *Appl. Microbiol. Biotechnol.* 29, 392 (1988).

17. J. M. Henson, M. V. Yates, J. W. Cochran, and D. L. Shackleford, *FEMS Microbiol. Ecol.* 53, 193 (1988).

18. J. M. Henson, M. V. Yates, J. W. Cochran, *J. Ind. Microbiol.* 4, 29 (1989).

19. G. W. Strandberg, T. L. Donaldson, and L. L. Farr, *Environ. Sci. Technol.* 23, 1422 (1989).

20. N. A. Lanzarone and P. L. McCarty, *Ground Water* 28, 910 (1990).

21. S. E. Strand, M. D. Bjelland, and H. D. Stensel, *Res. J. Water Pollution Control Fed.* 62, 124 (1990).

22. K. Broholm, B. K. Jensen, T. H. Christensen, and L. Olsen, *Appl. Environ. Micobiol.* 56, 2488 (1990).

23. S. M. Henry and D. Grbic-Galic, *Appl. Environ. Microbiol.* 57, 236 (1991).

24. L. Alvarez-Cohen and P. L. McCarty, *Appl. Environ. Microbiol.* 57, 228 (1991).

25. L. Alvarez-Cohen and P. L. McCarty, *Appl. Environ. Microbiol.* 57, 1031 (1991).

26. C. D. Little, A. V. Palumbo, S. E. Herbes, M. E. Lidstrom, R. L. Tyndall, and P. J. Gilmore, *Appl. Environ. Microbiol.* 54, 951 (1988).

27. H.-C. Tsien, G. A. Brusseau, R. S. Hanson, and L. P. Wackett, *Appl. Environ. Microbiol.* 55, 228 (1989).

28. R. Oldenhuis, R. L. J. M. Vink, D. B. Janssen, and B. Witholt, *Appl. Environ. Microbiol.* 55, 2819 (1989).

29. S. M. Henry and D. Grbic-Galic, *Microbial Ecol.* 20, 151 (1990).

30. G. A. Brusseau, H.-C. Tsien, R. S. Hansen, and L. P. Wackett, *Biodegradation* 1, 19 (1990).

31. R. Oldenhuis, J. Y. Oedzes, J. J. van der Waarde, and D. B. Janssen, *Appl. Environ. Microbiol.* 57, 7 (1991).

32. H. Dalton, *Adv. Appl. Microbiol.* 26, 71 (1980).

33. C. T. Hou, *Appl. Microbiol. Biotechnol.* 19, 1 (1984).

34. D. Henschler, W. R. Hoos, H. Fetz, E. Dallmeier, and M. Metzler, *Biochem. Pharmacol.* 28, 543 (1979).

35. R. Miller and F. P. Guengerich, *Biochemistry* 21, 1090 (1982).

36. E. C. Donaldson, G. V. Chilingarian, and T. F. Yen, Eds., *Microbial Enhanced Oil Recovery*, Elsevier Press (1989).

37. (a) S. Park, M. L. Hanna, R. T. Taylor, and M. W. Droege, *Biotech. Bioeng.* 38, 423 (1991); (b) S. Park, N. N. Shah, R. T. Taylor, and M. W. Droege, *Biotech. Bioeng.*, in press (1992); (c) N. N. Shah, S. Park, R. T. Taylor, and M. W. Droege, *Biotech. Bioeng.*, submitted (1992). The *M. trichosporium* OB3b bacteria were batch cultivated in a 5-L bioreactor on minus-$CuSO_4$ Higgin's minimal nitrate salts medium that was fortified with 2x $FeSO_4 \cdot 7H_2O$ and 2x nitrate as recommended previously for forming exclusively intracellular sMMO (37a). However, to enhance the resting-state storage stability of this cellular sMMO, for the test bed TCE biodegradation experiments in Figs. 2-7 (this paper), the culture medium $NaMoO_4 \cdot 2H_2O$ was raised 20x to 8 μM and $NiCl_2$ was present at 7.5 μM. After 80 hours of culturing, additional 1x amounts of both $MgSO_4 \cdot 7H_2O$ and $FeSO_4 \cdot 7H_2O$ were added to the cultures; the cells were harvested after 95 hours at the end of their transition from a fast to a slow growth rate imposed by nitrate depletion in the culture medium.

38. Bacterial cells were enumerated with a Coulter electronic particle counter (model ZB1) having a 30 μm aperture; and the particles counted were verified to be bacteria by their size distribution, which was determined with a Coulter Channelizer and x-y recorder. The counting fluid was 5.0 ml of triple-filtered 4% NaCl.

39. R. T. Taylor and M. L. Hanna, unpublished data.

40. TCE concentrations were determined by gas chromatography, according to the U.S. EPA test method no. 601 for volatile halogenated organic compounds, Fed. Reg. 49, No. 209, Oct. 26 (1984). Aqueous (1 ml) samples were removed from the test bed via the attached ports (see Fig. 1a), placed in glass purging vials, and then stored in a refrigerator several hours until the

analyses could be performed. The analyses were carried out with a Hewlett-Packard model 5880 gas chromatograph using a J & W DB-624 capillary column (30 m x 0.5 mm i.d.). It was equipped with an electron capture detector, an H-P model 3396 integrator, a Dynatech Precision PTA-30 W/S auto-sampler, and an O. I. Analytical model 4460A purge and trap concentrator. The detection limit for TCE was 0.5 ppb.

41. H. S. Carslaw and J. C. Jaeger, *Conduction of Heat in Solids*, Clarendon Press (1959).

42. Reaction mixtures (1.0 ml) containing stored resting cells and [1,2-^{14}C]TCE in 10 mM Higgin's phosphate buffer were incubated at room temperature in 5-mL amber vials that were sealed with open-top-closure screw caps and PTFE-faced rubber septa. At the times indicated, the reactions were terminated by injecting 50 µL of 1.0 M $NaHCO_3$ and 1.0 ml of unlabeled TCE. The sealed vials were centrifuged 1 minute at top speed in a clinical centrifuge to separate the aqueous and organic liquid phases. A sample (0.6 mL) of the upper aqueous phase was removed and its radioactivity was determined with 10 mL of ICN Universol counting fluid in a Packard Tri-Carb liquid scintillation spectrometer. [1,2-^{14}C]TCE (13.1 µCi/µmol) was purchased from the Sigma Chemical Co. and adjusted to the desired specific radioactivity and stock concentration (0.25 mM) with unlabeled TCE (99+% obtained from the Aldrich Chemical Co.)

43. We acknowledge the valuable technical input and assistance of D. J. Bishop, C. Boro, G. Cobb, F. Holdener, K. Keller, S. Martin, R. Martinelli, S. Nakano, W. Ralph, D. Ruddle, C. Steffani, J. Ueng, and B. Viani in the design and construction of the 1.1-m test bed system and in the collection of the TCE samples. This work was performed under the auspices of the U. S. Department of Energy (DOE) by Lawrence Livermore National Laboratory under Contract 7405-Eng-48. The work was supported by the DOE Office of Technology Development under the direction of Deputy Assistant Secretary Dr. C. Frank, Office of Environmental Restoration and Waste Management.

FIGURE LEGENDS

Figure 1. (a) Schematic of the 1.1-m test bed showing the flow direction and locations of the 75 fluid sampling ports (black dots). Bacteria (4.7×10^9 cells/mL) were injected into the sand test bed through the middle column of sampling ports in the left-hand plate (Plate I) at a rate of 2 mL/min/port using a peristaltic pump. Fluid was simultaneously removed, at 1 mL/min/port, from the second and fourth columns of ports (second peristaltic pump) to establish a closed circulatory system. The peristaltic pumping directions were then reversed and the zone between the second and fourth column of ports was flushed with 10 mM Higgin's medium phosphate buffer (pH 7.0) (37) to remove the unattached cells. During the bacterial injection and withdrawal procedure, the ambient flow field was stagnant. From the difference between the number of cells injected and the number withdrawn, the number of bacteria that initially attached in the zone was determined to be 1.6×10^{12} (38). Establishment of the microbial zone and all of the subsequent flow-through experiments were carried out at 21-22°C. (b) Photograph of the sealed operational test bed system, viewed from the opposite side and showing the glass windows. Also shown is the top assembly, which serves to seal the upper surface of the sand pack.

Figure 2. Experimental data and simulation (for no biodegradation) of the TCE wave at the sampling port in the upper left corner of Plate I (Fig. 1a), immediately upstream from the microbial filter, during the first experiment. C/C_0 is the concentration relative to the input 109-ppb TCE pulse (40). Travel time shown on the x-axis in this and subsequent figures is relative to the start of flow for a TCE wave; it can be related to position via the 1.5-cm/h flow velocity.

Figure 3. Experimental data and simulation (for no biodegradation) of the TCE wave at the sampling port in the upper right corner of Plate I, immediately downstream from the microbial filter, during the first experiment. C/C_0 is the concentration relative to the input 109 ppb-TCE pulse (40).

Figure 4. Experimental data and simulation (for no biodegradation) of the TCE wave at the sampling port in the lower left corner of Plate I, immediately upstream from the microbial filter, during the second experiment. C/C_0 is the concentration relative to the input 85-ppb TCE pulse (40).

Figure 5. Experimental data and simulation (for no biodegradation) of the TCE wave at the sampling port in the lower right corner of Plate I, immediately downstream from the microbial filter, during the second experiment. C/C_0 is the concentration relative to the input 85-ppb TCE pulse (40).

Figure 6. Comparison of the TCE mass balance data from the control experiment (469 μg pulse), generated prior to the injection of bacteria, with the data from the first biofilter experiment collected following establishment of the microbial filter zone. Data are from samples taken along the middle row (Row C) in each plate of the 1.1-m test bed. Row C is about 0.2 m from the bottom of the sand pack. The port at location I-C-1 is approximately 0.1 m from the inlet port; the other locations, all with respect to the inlet are: II-C-1 ~0.45 m, II-C-5 ~0.65 m, and II-C-5 ~0.99 m. The emplaced microbial filter was located between 0.16 and 0.25 m.

Figure 7. Measurements of the attached microbial population (top) in millions of attached cells/g of dry sand, and their residual sMMO [1,2-^{14}C]TCE biotransformation activity (bottom), in nmol/min/g of dry sand in Plate I. Black dots show the location of the sampling ports in Plate I for reference. These data were obtained following the two experiments with the emplaced biofilter, 17 days after the bacteria were originally injected. The sand pack was sequentially excavated in six horizontal layers between the removable fritted nickel tubes. Ten wet sand samples per layer (each containing 2.1 g of dry sand, taken from the center of the pack across Plate I) were analyzed for their cell number and their steady-state [1,2-^{14}C]TCE bioactivity. These initial steady-state rate assays were performed at 30°C in the presence of formate, by replacing propene with 0.5 μmole of [1,2-^{14}C]TCE (2,000 cpm/nmol) in our standard (0.5 mL) whole-cell sMMO incubation mixture (37). The lower limit for measuring the rate of radiolabeled TCE degradation in these assays is 0.02 nmol/g/min. The attached cells were

readily dislodged by shaking the wet sand samples for 30 seconds in 5 mL of Higgin's phosphate buffer. After allowing the sand particles to settle, aliquots of the suspended bacteria were removed for cell counting (38) and [1,2-^{14}C]TCE activity assays.

Figure 8. Whole-cell biotransformation of a [1,2-^{14}C]TCE concentration similar to the unlabeled TCE pulses injected into the 1.1-m test bed for the biofilter experiments. An aliquot of the same cell suspension that was used to create the test bed microbial filter was stored 17 days at room temperature in Higgin's phosphate buffer (pH 7.0). Samples, each containing 5 x 10^8 cells, were then incubated in 1.0-mL volumes of Higgin's phosphate buffer with 1.0 nmol (131 ppb) of [1,2-^{14}C]TCE (4,000 cpm/nmol) and the amount of bioconversion was determined at the times shown (42).

Figure 9. Resting-state cell storage of sMMO-containing bioreactor-grown *M. trichosporium* OB3b. The cells were batch-cultivated in minus-$CuSO_4$-modified Higgin's medium as described (37), harvested and washed, and then stored in 10 mM Higgin's phosphate buffer (pH 7.0) as a 1.0 mg/mL cell suspension in stoppered 2-mL plastic tubes at room temperature. At the times shown, cell suspension samples were removed and assayed at 30°C for their initial steady-state rates of [1,2-^{14}C]TCE (2,000 cpm/nmol) biotransformation, in the presence of formate, by substituting 0.5 μmol of labeled TCE for propene in a standard whole-cell sMMO incubation mixture (37).

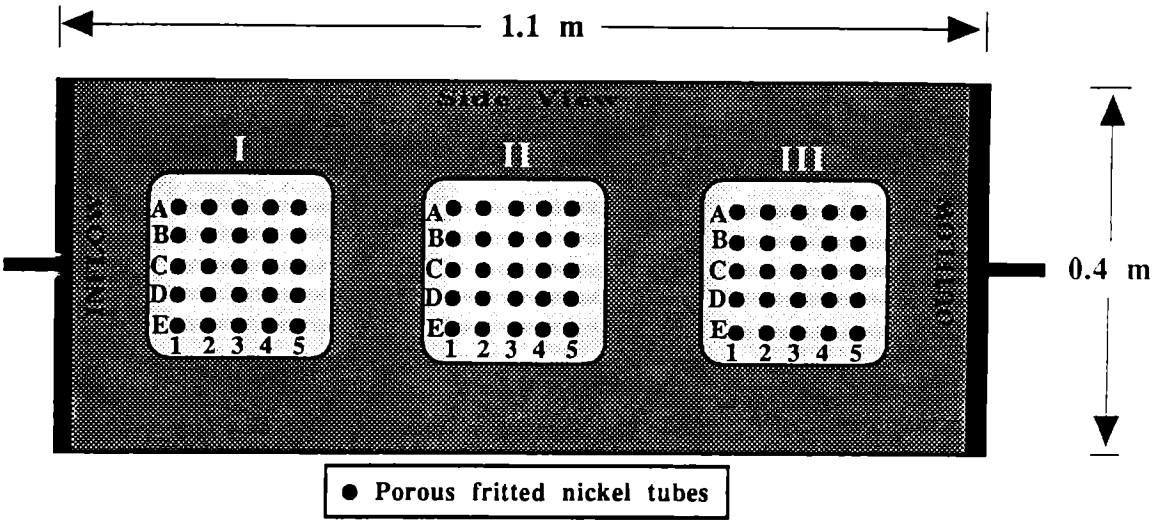

1.1 m
Side View
I
II
III
A
B
C
D
E
1 2 3 4 5
INLET
OUTLET
0.4 m
Porous fritted nickel tubes

Fig. 1a

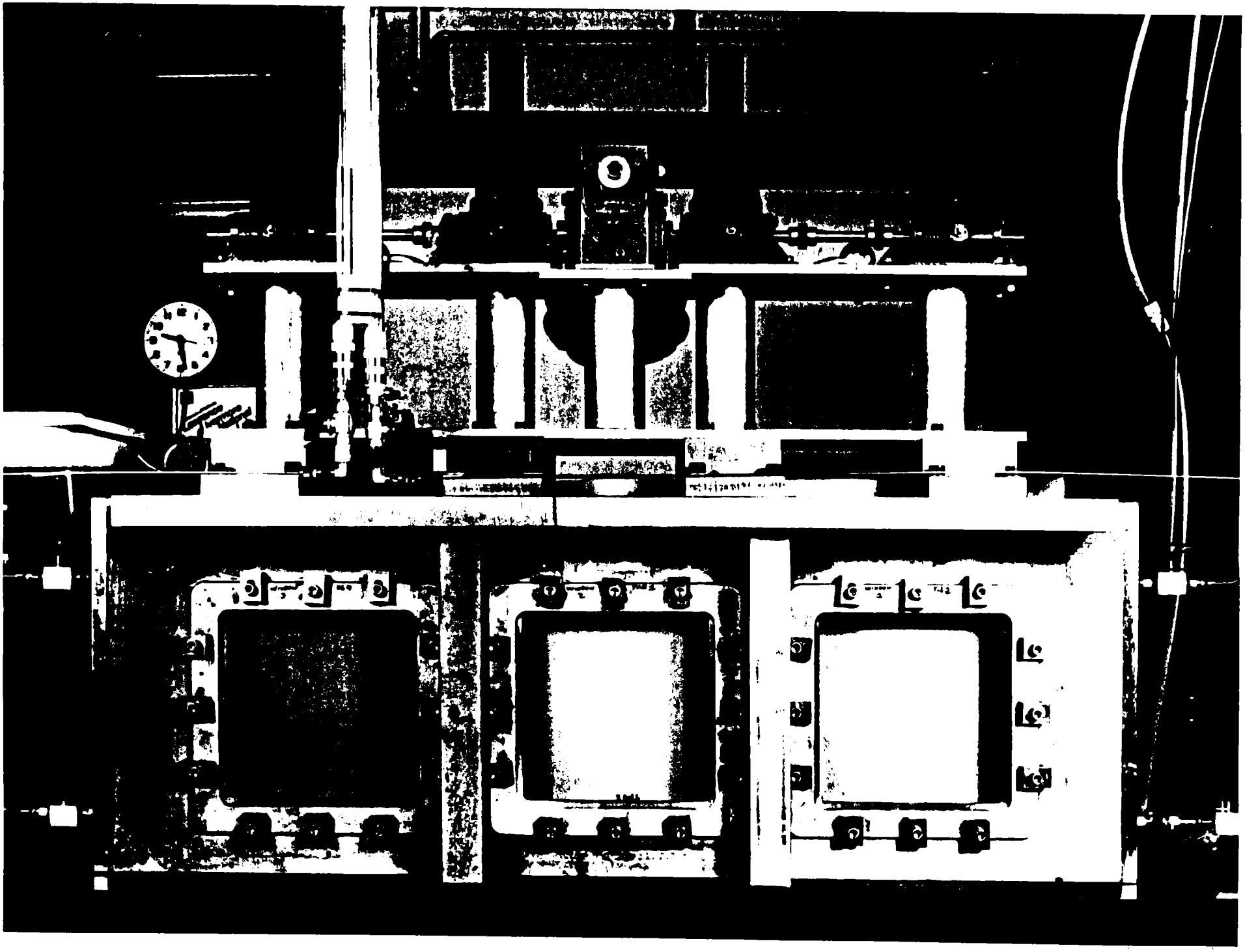


Fig. 1b

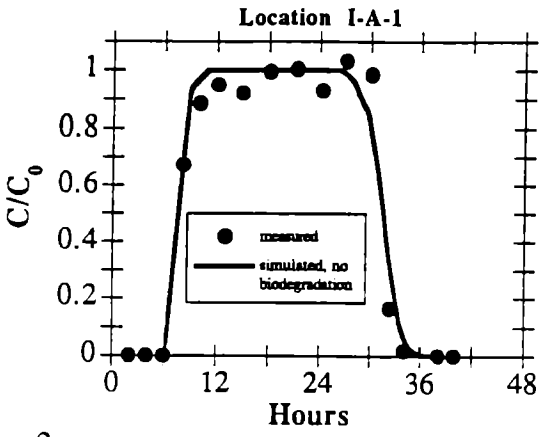


Fig. 2

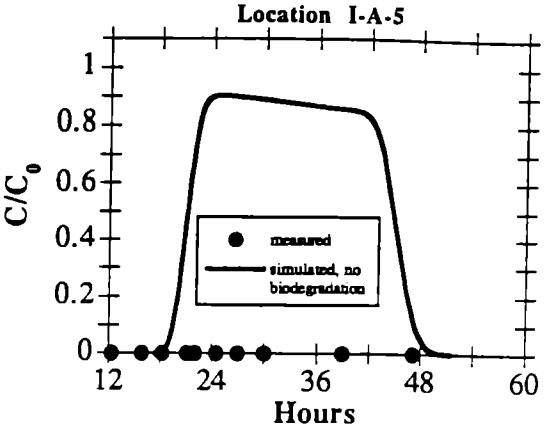


Fig. 3

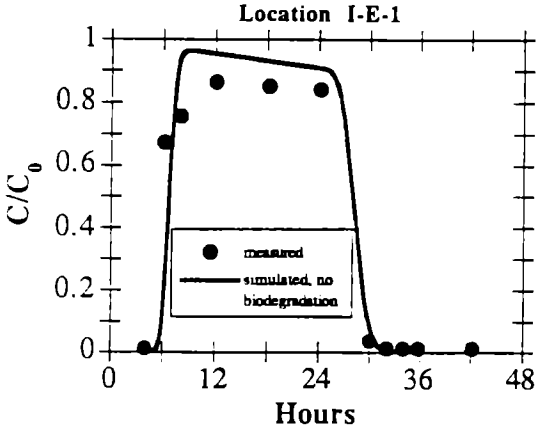


Fig. 4

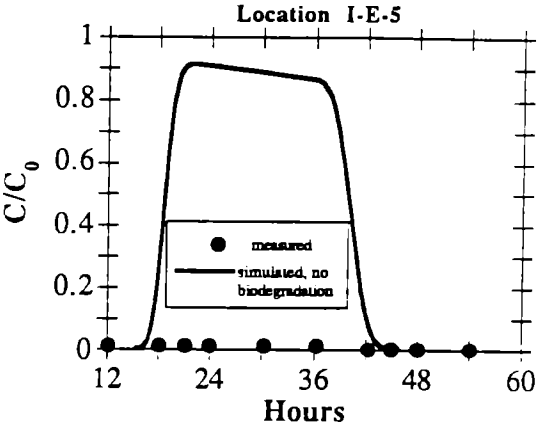


Fig. 5

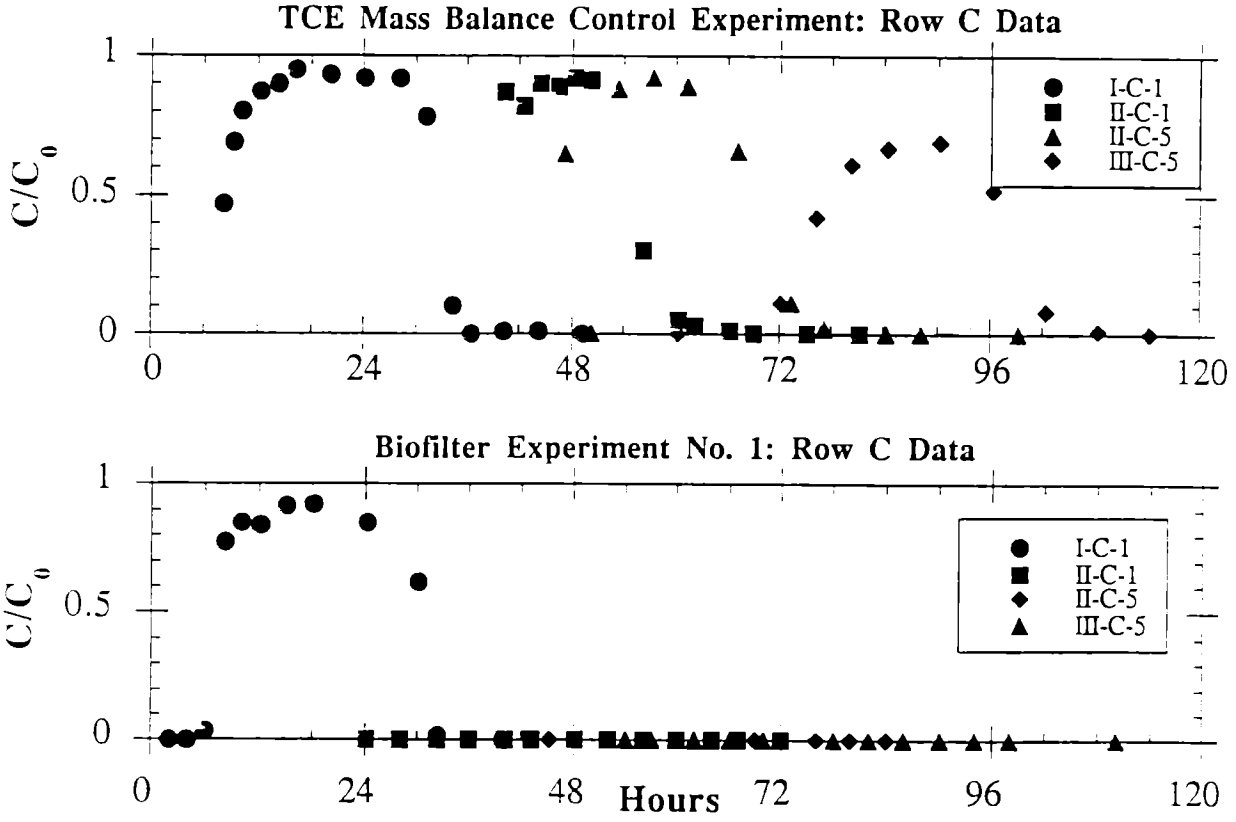


Fig. 6

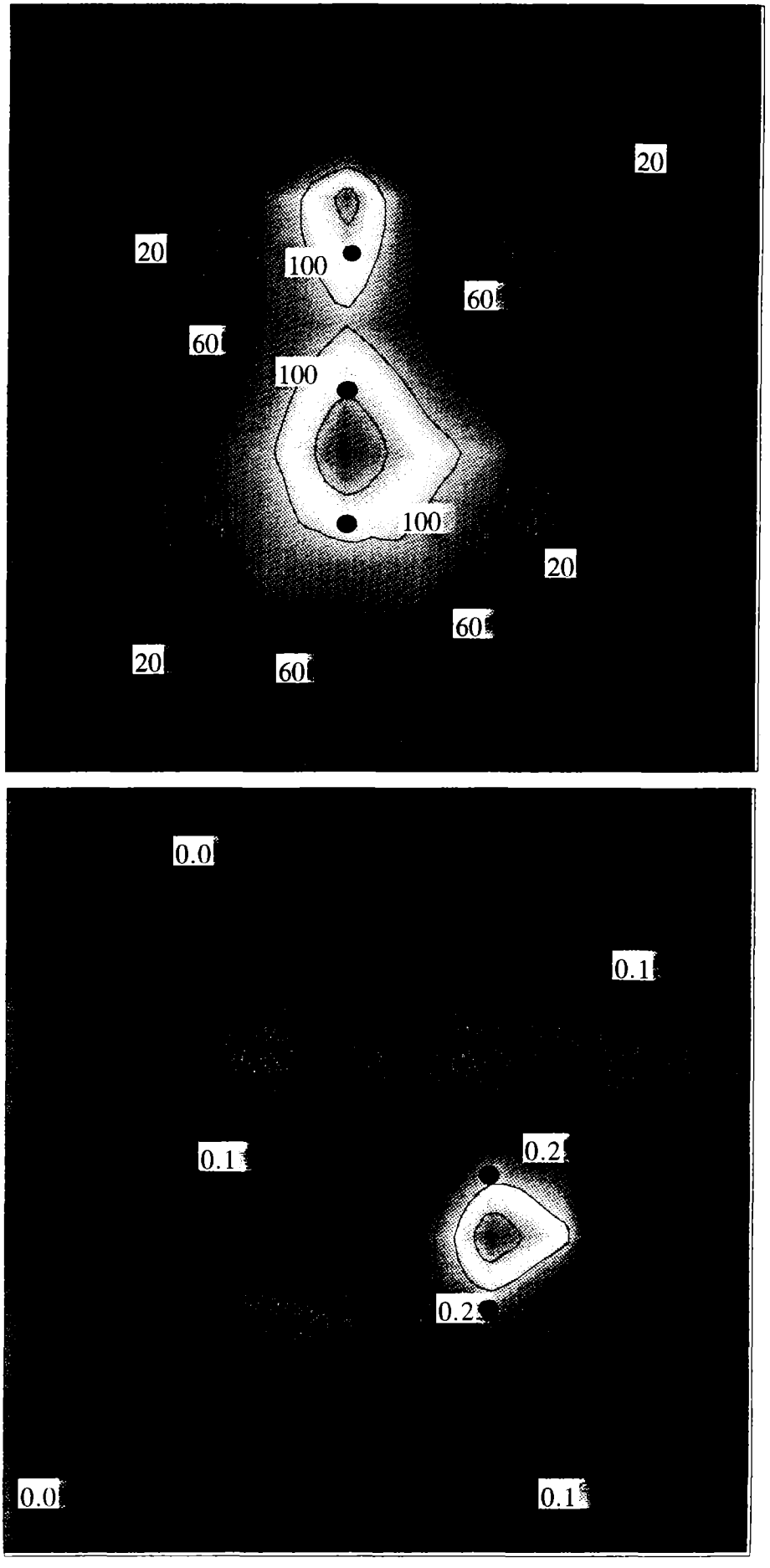


Fig. 7

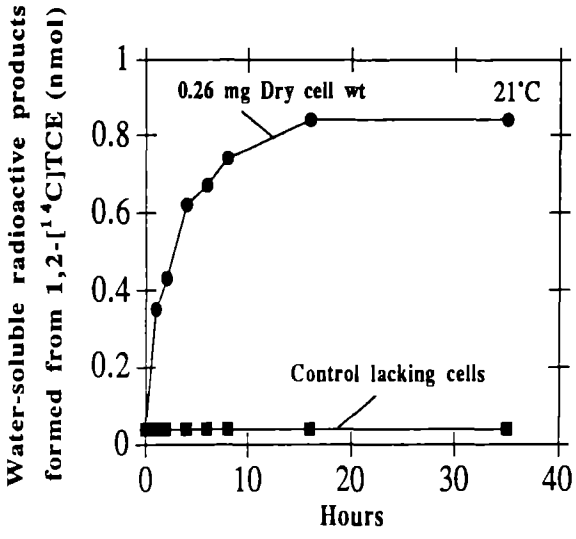

Water-soluble radioactive products formed from 1,2-[14C]TCE (nmol)
0.26 mg Dry cell wt
21°C
Control lacking cells
Hours

Fig. 8

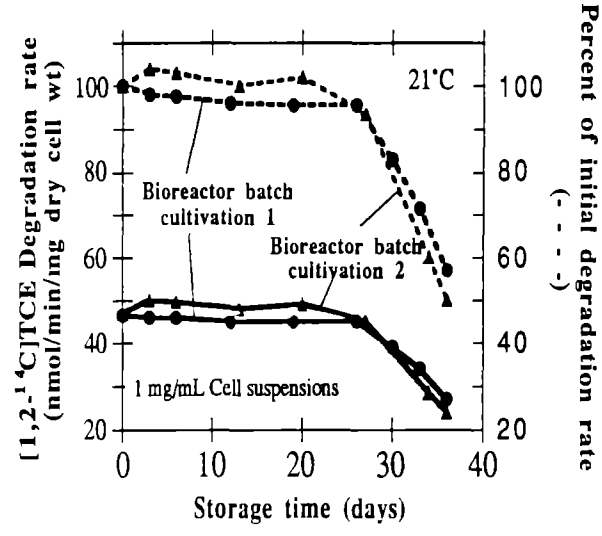

[1,2-14C]TCE Degradation rate (nmol/min/mg dry cell wt)
Percent of initial degradation rate (- - - -)
21°C
Bioreactor batch cultivation 1
Bioreactor batch cultivation 2
1 mg/mL Cell suspensions
Storage time (days)

Fig. 9